FIXATION OF HCO_3^-

BY MALIC ENZYME REACTION

WITH COENZYME REGENERATION

YOKO OHNO

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PREFACE

This is the thesis submitted to University of Fukui by the author for the doctoral degree of Engineering. The studies collected here have been carried out in Whatman Japan Ltd. (now a part of GE Healthcare in Japan) and Nihon Cytec Industries Ltd. during 2004 – 2008.

The studies were carried out under the guidance of Associate Professor Shin-ichiro Suye at Department of Applied Chemistry and Biotechnology, Faculty of Engineering, University of Fukui. The author greatly thanks to the sincere instructions to perform this study.

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Yoko Ohno

Fiber Amenity Engineering,

Graduate School of Engineering,

University of Fukui

CONTENTS

ABBREVIATIONS

G6P : glucose-6-phosphate

G6PDH : glucose-6-phosphate dehydrogenase

HEPES : N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid

MES : 2-morpholinoethanesulfonic acid, monohydrate

DH : dehydrogenase

LiPDH : lipoamide dehydrogenase

Alg-Na : sodium alginate

Alg-NAD^+ : NAD^+ bound in polyalginate

MV^+ : methyl viologen

Alg-V : viologen bound in polyalginate

Chapter 1 General introduction

Effective conversion of atmospheric CO_2 to industrially useful chemicals proposes promising new technology to generate carbon source alternatives as well as recycling of the green house gas. Petrochemical industry discharges abundant CO_2 which is not recycled yet. In the past, hazardous wastes and drains from chemical industry caused environment pollution but nowadays some solvents were recycled or well-treated and some are decomposed to CO_2 and water (Fig. 1).

Major carbon sources of chemical products are oil and natural gas. They are also used as energy source. Oil price is not always stable due to the balance between supply and demand in the market. Material cost escalation is big issue in chemical industry. Another issue is new regulation of CO_2 emission. In this chapter recent material cost trend and environmental CO_2 issues are reviewed then technical approaches of fixation of CO_2 gas or aqueous HCO_3^- are reviewed to discuss efficient CO_2 recycle technology .

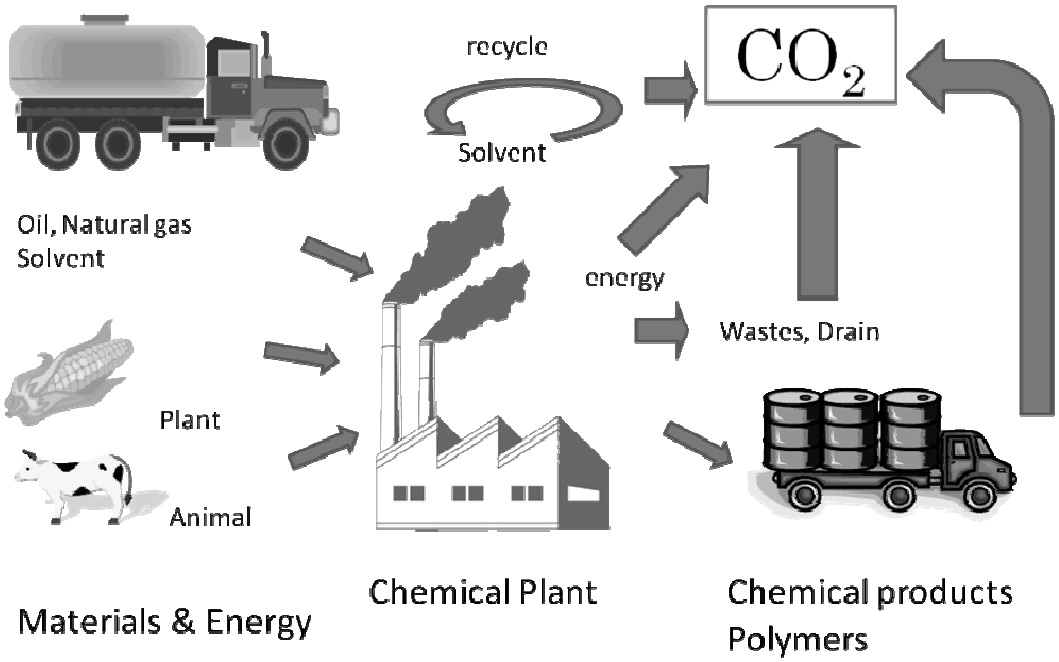

Fig. 1 CO_2 discharge at petrochemical industry

Material cost and supply

The price increase of oil influences on not only chemical industry but on global economy. Oil crises of the 1970s resulted in inflation thereafter. In those few years the price of oil and natural gas escalated significantly and chemical price followed it as shown in Fig.2. Oil price escalation influenced on the price of crops because Energy Law in US in 2005 obliged to replace oil with up to 7.5 gallons of biomass energy in 2012. The established technology of biomass at industrial level in US was ethanol from maize, called

bioethanol. When oil price escalated the maize price increased then the price of the other crops like wheat was also raised (Fig. 2).

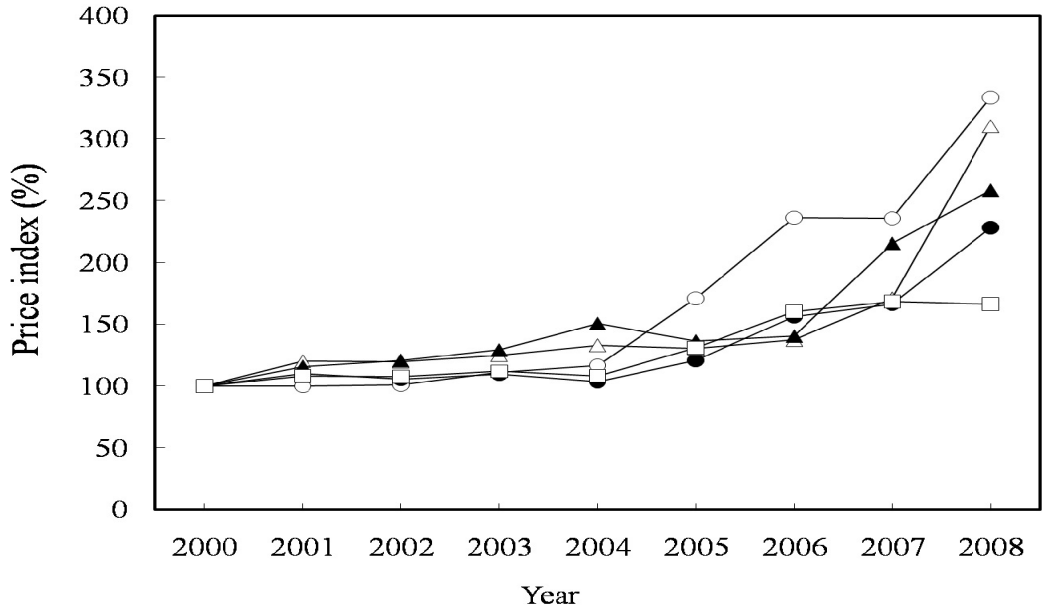

Fig. 2 Material cost trend from year 2000 to 2008.
The data was from trade index of import value in Trade Statistics of Japan, Ministry of Finance (http://www.customs.go.jp/toukei/download/index_p03_e.htm). The index is based on import price of year 2000 when wa 100. Import price index of crude oil (○), natural gas (●), wheat (△), maize(▲) and organic chemical (□)

Alternative energy source of oil was being developed and used, for example, hydroelectric energy, nuclear energy, photovoltaic generation, wind power generation,

biomass energy etc. (NEDO http://www.nedo.go.jp/introducing/about.html). An alternative carbon source for use in industry with stable supply and with stable cost is discussed in this paper focusing on efficiency of CO_2 recycle use. First, atmospheric CO_2 trend and issue were surveyed. Next, the technical approaches of cost-effective CO_2 use were investigated in Chapter 1.

Atmospheric CO_2

Atmospheric CO_2 is increasing which is one of the major factors causing green house effect (Fig. 3) .

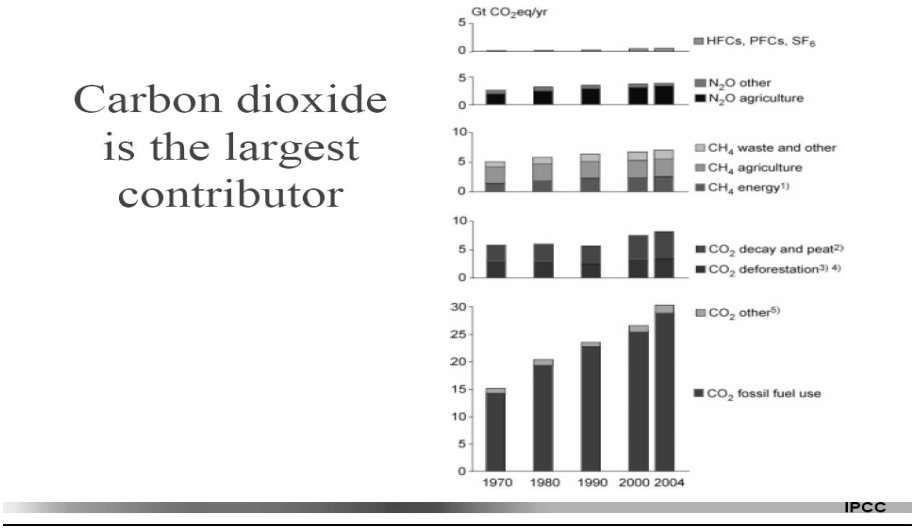

Fig. 3 Atmospheric CO_2 as largest contributor of green house effect (IPCC 2005) (1)

It is reported that CO_2 contributes about 50% of the greenhouse gas effect, and its concentration in the atmosphere is continuously increasing (2-4). Recently it is socially required to reduce carbon dioxide because of global warming issue (1).

Carbon recycle

In nature world direct use of CO_2 or HCO_3^-, which is mediated by enzymes widely occurs and carbon dioxide is well recycled as shown in Fig.4. It was said that around 60% of CO_2 was converted to HCO_3^- and recycled in water.

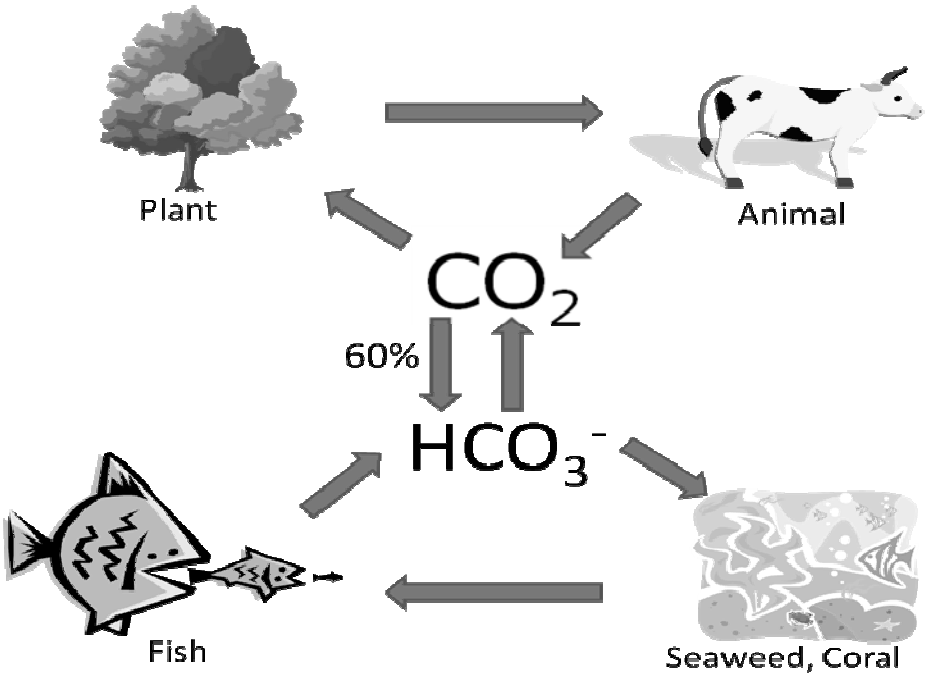

Fig. 4 Recycle of carbon in nature world

On the other hand CO_2 is not recycled in chemical industry and abundant CO_2 is generated mainly from oil or natural gas. Cost-effective industrial technology to utilize aerial CO_2 or HCO_3^- in water is limited since appropriate technology is not developed yet.

There have been several attempts to fix CO_2 or HCO^-_3 with physical and chemical processes. Aresta et. al. reviewed direct use of CO_2, in which two processes were referred to, namely, Kolbe-Schmitt reaction to produce benxoic acid, and urea synthesis by Fumasoni et. al. 1974 and Fromm et al 1979, which were successfully industrialized, but the yields were not high. Aresta.et.al. analyzed energy level of CO_2 and found that it was lower than that of organic chemicals. So any use of CO_2 requires energy, which made direct carboxylation difficult. They recommended using enzyme reaction to overcome the energy gap. (5)

Several trials were reported to fix CO_2 in useful products chemically(6,7), electrochemically(8,9), photochemically(10,11), and enzymatically (8,9,12-19) such as fixation of CO_2 in oxoglutaric acid by isocitrate dehydrogenase and in pyruvic acid by malic enzyme. Malic enzyme reaction favored a forward reaction for decarboxylation of L-malic acid with NAD^+ reduction (18, 19).

Malic enzyme and NAD(P)/NAD(P)H regeneration

Malic enzyme catalyzed the decarboxylation of L-malic acid to pyruvic acid and at the same time release CO_2 from malic acid by using oxidized form of coenzyme, $NAD(P)^+$ as electron acceptor. The reverse reaction was used for fixation of CO_2 by Sugimura et al. (8, 9). In the reverse reaction, pyruvic acid is converted to L-malic acid by CO_2 or HCO_3^- and NAD(P)H, reduced form of coenzyme, is oxidized to $NAD(P)^+$（Fig.5）.

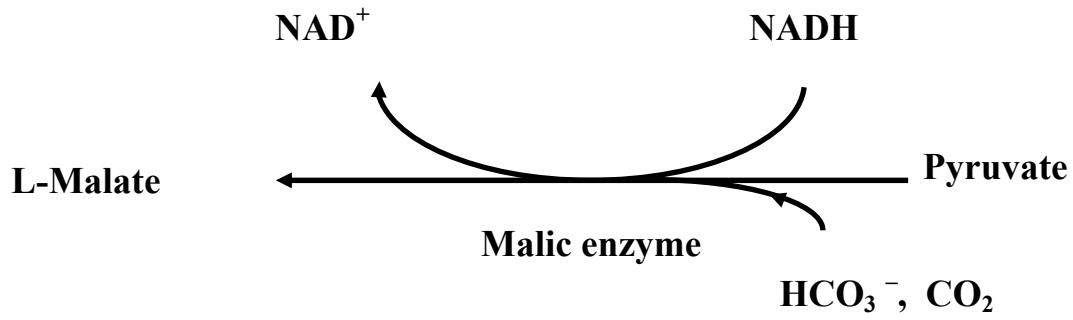

Fig. 5 Malic enzyme reverse reaction

In biosynthesis the reduction of $NAD(P)^+$ to generate NAD(P)H is essential to promote the reverse reaction of malic enzyme(20). It is applicable for biosensing system

with electrochemical NAD(P)H regeneration and detection of $NAD(P)^+$ with high efficiency (20-22). Regeneration of NAD(P)H by both biochemical and electrochemical methods were reported (23-26), and electrode modified by layer-by-layer adsorption (LBL) of polymerized mediator and polymerized coenzyme also exhibited NAD(P)H regeneration performance (27,28).

Biochemical or electrochemical $NAD(P)^+$/NAD(P)H reduction is required for malic enzyme reverse-reaction. In Chapter 2 the efficient fixation of HCO_3^- and CO_2 was studied with malic enzyme reverse-reaction using enzymatic NADH regeneration system. In Chapter 3 electrochemical - enzymatic NADH regeneration system with LBL method was studied to fix HCO_3^- cost effectively using malic enzyme reverse-reaction.

Chapter 2 Reverse reaction of malic enzyme for HCO_3^--fixation into pyruvic acid to synthesize L-malic acid with enzymatic coenzyme regeneration

Introduction

Reverse reaction of malic enzyme has been designed and used for fixation of HCO_3^- or CO_2 with coenzyme regeneration such as photochemical or electrochemical reaction (11, 29-31).

Suye et al. found that *Pseudomonas diminuta* IFO13182 produced malic enzyme (EC 1.1.1.39) and investigated its applications, focusing on production of a reduced form coenzyme (21, 23,24). L-alanine was produced from malic acid and NH_4^+ combining with malic enzyme and alanine dehydrogenase with NADH regeneration system (21) as shown in Fig 6.

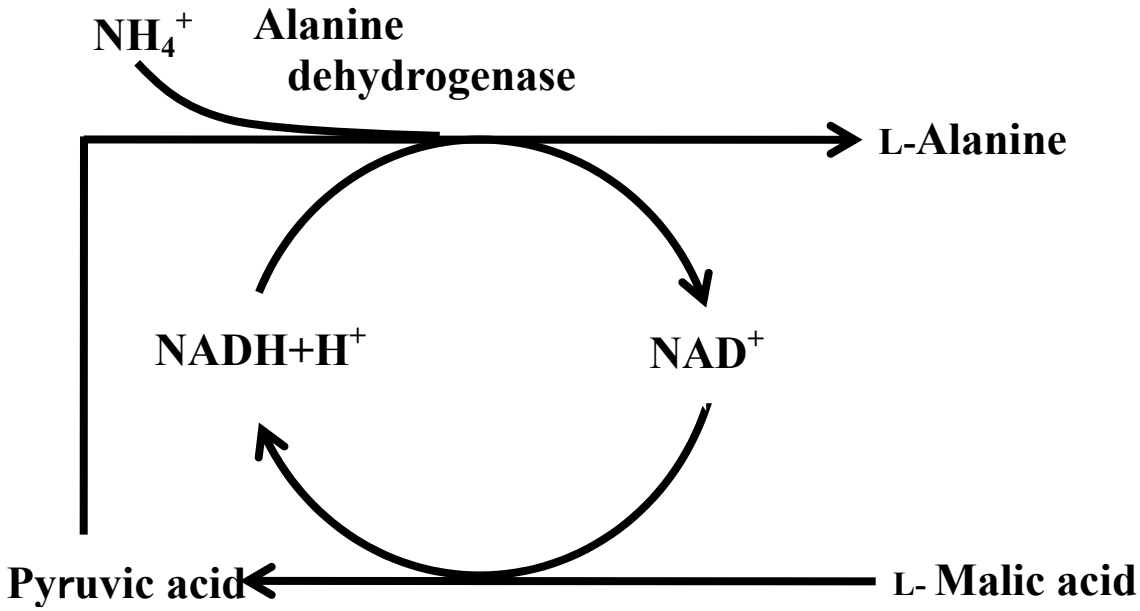

Fig. 6 Schematic illustration of L-alanine production combined with malic enzyme and alanine dehydrogenase with NADH regeneration system by Suye et al. (21)

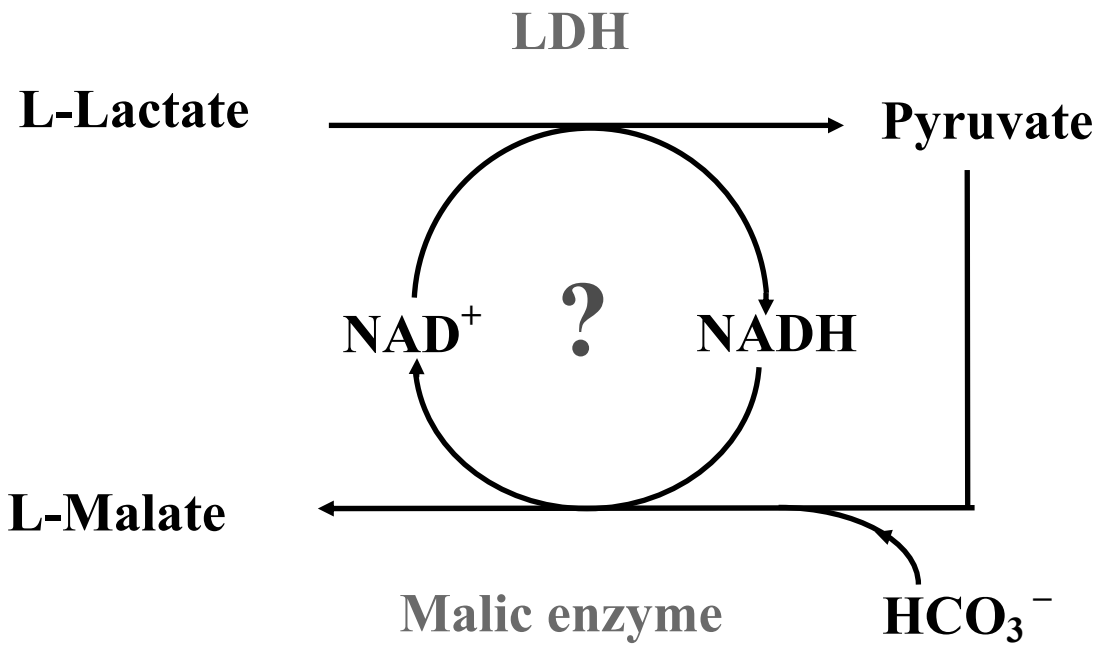

Fig. 7 Design of one substrate system to fix HCO_3^- (32)

As regenerators of NAD(P)H several enzymes are known, eg. alcohol dehydrogenase, Ferredoxin-NADP reductase, Formate dehydrogenase, glucose-6-phoshate dehydrogenase (G6PDH), hydrogenase, lactose dehydrogenase (LDH), galactose dehydrogenase and malate dehydrogenase. (33)

One substrate system to fix HCO_3^- with malic enzyme reverse reaction and with lactate dehydrogenase (32,33) was tested initially (Fig. 7). Then HCO_3^- fixation as well as CO_2 (gas) by the reverse reaction of malic enzyme with NADH regeneration using G6P dehydrogenase forward reaction (34,35) was designed as shown in Fig. 8. Furthermore NAD^+ was immobilized on water-soluble polymer for repeat use of NAD^+ (36).

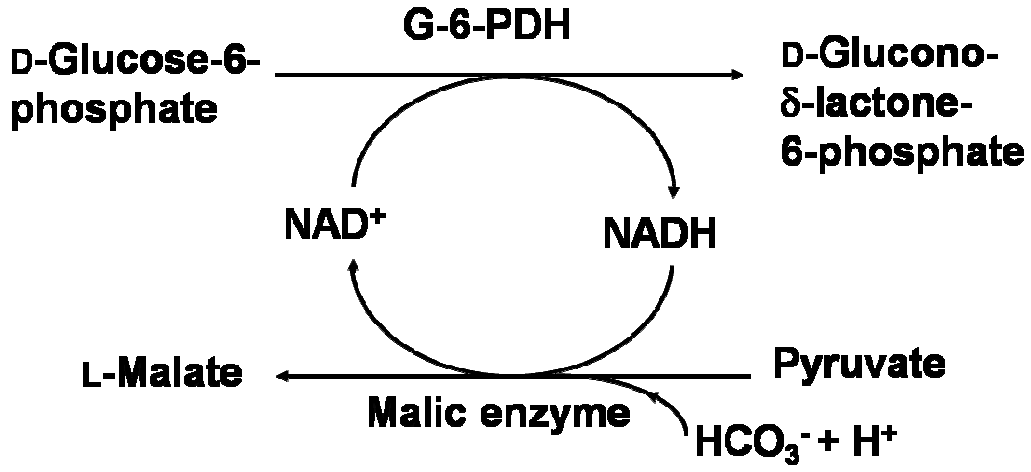

Fig. 8 Design of malic enzyme reverse reaction with coenzyme regeneration conjugated with G6PDH forward reaction.(34,35)

Finally fixation of CO_2 bubbling gas in water into malic acid was also tested replacing HCO_3^- in the system to find wheather CO_2 is usable in this enzymatic system.

Materials and Methods

Chemicals

Glucose-6-phosphate dehydrogenase (G6PDH, EC1.1.1.49, 400 units·mg^{-1}) of *Leuconostoc mesenteroides* was obtained from Oriental Yeast Co., (Tokyo, Japan). Pig heart lactate dehydrogenase of (EC1.1.1.27, 350 units·mg^{-1}) was obtained from Toyobo Co., (Tokyo, Japan). NAD^+ was obtained from Oriental Yeast Co. N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 2-morpholinoethanesulfonic acid, monohydrate (MES) were obtained from Nacalai Tesque Co., (Kyoto, Japan). The other reagents and compounds were analytical grade and used without further purification.

Preparation of malic enzyme

Pseudomonas diminuta IFO 13182 was cultured in a 30 liter jar-fermenter and prepared as a cell-free extract with partial purification as described previously (36). Malic enzyme was purified 44-fold over the cell-free extract with final yield of 49% and specific activity of 0.989 units·mg^{-1} protein.

Assay of malic enzyme activity for reverse and forward reactions

The enzyme activity for forward reaction was measured as previously described (24). The reverse reaction was measured in a $KHCO_3$ solution as a carbon source *via* the NADH-conversion of L-malic acid from pyruvic acid. The activity was measured spectrophotometrically by measuring the decrease in the absorbance of NADH at 340 nm. The substrate mixture contained 60 μmol of pyruvic acid, 30 μmol of $MgCl_2$, 0.6 μmol of NADH, 60 μmol $KHCO_3$, and 100 μmol of HEPES-KOH buffer, pH 7.4, in a total volume of 2.0 ml. The reaction was started by adding 1.0 ml of the enzyme solution and incubating at 30ºC. After exactly 2 min, 1.0 ml of 10% (w/v) sodium dodecyl sulfate (SDS) was added to stop the reaction and the absorbance at 340 nm was measured. In a blank test, 10% SDS

was added before the enzyme solution. One unit of activity was defined as the amount of the enzyme that produced one μmol of NADH per min at 30ºC. In calculating the amount of NADH produced, a molar absorption coefficient for NADH of 6.22 × 103 $M^{-1\cdot}$ ▪ cm^{-1} was used.

Synthesis of L-malic acid with coenzyme regeneration using G6PDH

Reverse reaction of malic enzyme with NADH regeneration using G6PDH was also carried out using glucose-6-phosphate (G6P) and G6PDH.

The standard reaction mixture for malic enzyme reverse reaction with NADH regeneration using G6PDH contained 1.0 μmol of NAD^+, 100 μmol of G6P, 100 μmol of pyruvic acid, 15 μmol of $MgCl_2$, 100 μmol $KHCO_3$, 0.5 unit of malic enzyme, and 0.5 unit G6PDH in 1.0 ml of 55 mM potassium phosphate buffer (pH 7.4). The reaction mixture was incubated at 30°C.

Preparation of water-soluble macromolecular polymer immobilized NAD^+

Water soluble polymer (sodium alginate) immobilized NAD^+ (Alg-NAD^+) was prepared as previously described (37). Samples of 13.8 mg sodium alginate and 70 mmol 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were dissolved in 15 ml water. The final pH was adjusted to 4.7 and the solution was stirred for 40 min at room temperature. NAD^+ was added to the solution to 70 mmol. After readjustment of the pH to 4.7, the resulting solution was stirred for 12 h at room temperature. The reaction mixture was dialyzed with 10 mM Tris-HCl buffer (pH 7.0) for 12 h and then dialyzed with water for 12 h at 5ºC. Alg-NAD^+ was recovered by lyophilization. Lyophilized products were stored at -20ºC in the dark. The coenzyme activity of Alg- NAD^+ was determined enzymatically by the alcohol dehydrogenase system (38).

HPLC analysis

HPLC analysis of L-malic acid, pyruvic acid, and L-lactic acid in the reaction mixture was performed using a Hitachi L-7100 and Shodex® Asahipak ES-502N (100 x 7.6 mm i.d.) (Showa Denko, Tokyo, Japan) at 50°C. The elution buffer consisted of 0.1 M potassium dihydorogenphosphate (KH_2PO_4) buffer (pH 4.2) at a flow rate of 1.0 ml·min^{-1}, at 50°C.

The detector was Hitachi U2010 ultraviolet/visible spectrophotometer (Hitachi, Tokyo, Japan) at 210 nm. Retention times of L-malic acid and pyruvic acid were 21.8 min and 11.1 min, respectively.

Results & Discussion

Reverse reaction activity of malic enzyme

The reverse reaction (carboxylation) and forward reaction (decarboxylation) activities of malic enzyme were measured at different pH. As shown in Fig. 9, optimum pH of reverse reaction was at pH 6.0 and carboxylation was strongly dependent on the pH value.

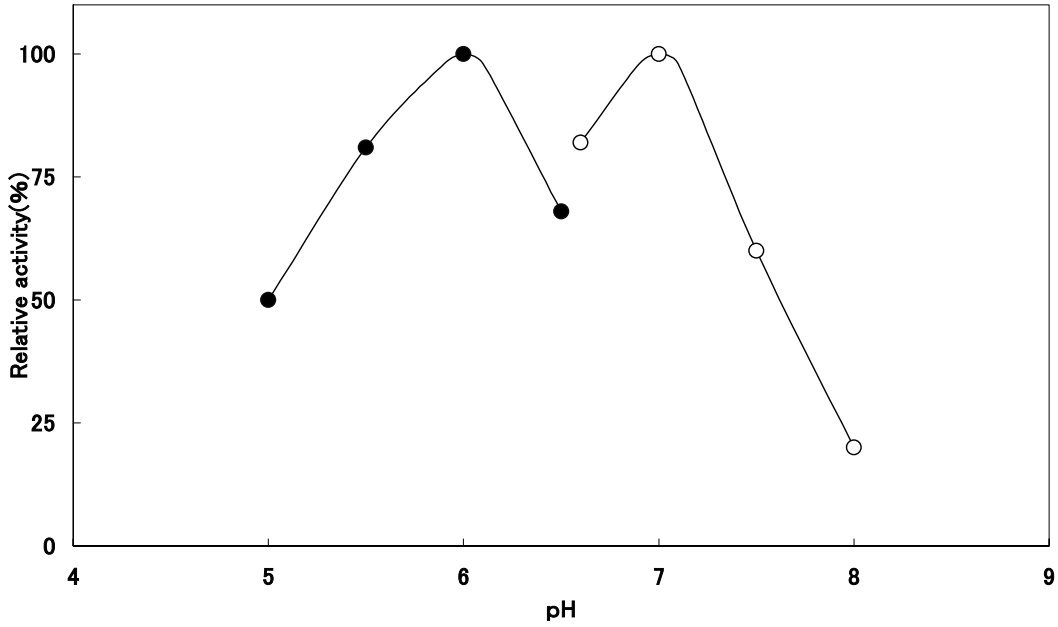

Fig. 9 Effect of pH on reverse reaction (carboxylation) activity of malic enzyme in MES buffer (●) and in HEPES buffer (○).The enzyme activity was measured at various pH 5–8.

However, reverse reaction activity was only 9.7 $\mu M \cdot min^{-1}$, representing only 5% of the forward reaction activity. Considering the results of the optimum pH of malic enzyme reverse reaction and stability of both reduced form (NADH) and oxidized form (NAD^+) coenzyme (39,40), pH 7.4 was used thereafter in malic enzyme reverse reaction mixtures.

Reverse reaction of malic enzyme with potassium ion was slightly better in terms of hydrogen carbonate fixation (5% of the forward reaction) than with sodium ion (2% of the forward reaction). $KHCO_3$ and potassium pyruvate were used for further investigation.

Reverse reaction of malic enzyme with coenzyme regeneration using G6PDH

Initially, LDH was used with malic enzyme reaction for NADH regeneration (32). However, L-malic acid, which is an indicator of hydrogen carbonate fixation into pyruvic acid in the reaction system, was not produced in the reaction mixture. The equilibrium of the LDH reaction for NADH regeneration is not favorable at a neutral pH region and L-lactic acid causes a decrease in the catalytic activities of the malic enzyme (11). LDH was not suitable for the regeneration reaction combined with reverse reaction of malic enzyme (Fig. 7).

Then two substrate systems were designed to improve the coenzyme regeneration. The G6PDH reaction was used as the NADH regenerator in the system since G-6-PDH is useful for regeneration of NADH from NAD^+ at neutral pH region (34, 35) (Fig.8).

The effects on malic enzyme reverse reaction were investigated changing conditions

The effects of NAD^+ concentration on malic enzyme reverse reaction are shown in Fig.10. Under the conditions of concentration of 0.5 and 1.0 mM NAD^+ in the reaction mixture, 39 to 40 mM L-malic acid was produced. Reaction mixture which contains 100 mM pyruvic acid and 0.5 mM NAD^+ was used for malic enzyme reverse reaction. The L-malic acid production was 38 mM after 24 h incubation (data not shown). The ratio of hydrogen carbonate and pyruvic acid to L-malic acid was about 38%. On the other hand, synthesis of L-malic acid combined with malic enzyme reverse reaction and photochemical coenzyme regeneration was reported. (31, 39) The ratio of hydrogen carbonate and pyruvic acid to L-malic acid was from 1.1 to 6.6%. In the case using 100 mM pyruvic acid and 0.5 mM NAD^+ in the reaction mixture was suitable for production of L-malic acid.

Reverse reaction of malic enzyme with Alg-NAD^+ as coenzyme

Suye et al. prepared Alg-NAD^+ coupling amino groups of NAD^+ and carboxy group of alginic acid with water soluble carbodiimide (36). Immobilization of coenzyme as well as enzyme is necessary for continuous or repeated use. Reverse reaction of malic enzyme with

coenzyme regeneration using Alg-NAD^+ as a coenzyme was also tested. As shown in Fig. 11, both two cases of Alg-NAD^+ concentration (equimolar of 0.5 and 1.0 mM free NAD^+) produced L-malic acid accumulated to about 33 mM. Alg-NAD^+ is also useful for L-malic acid production in the malic enzyme reverse reaction system.

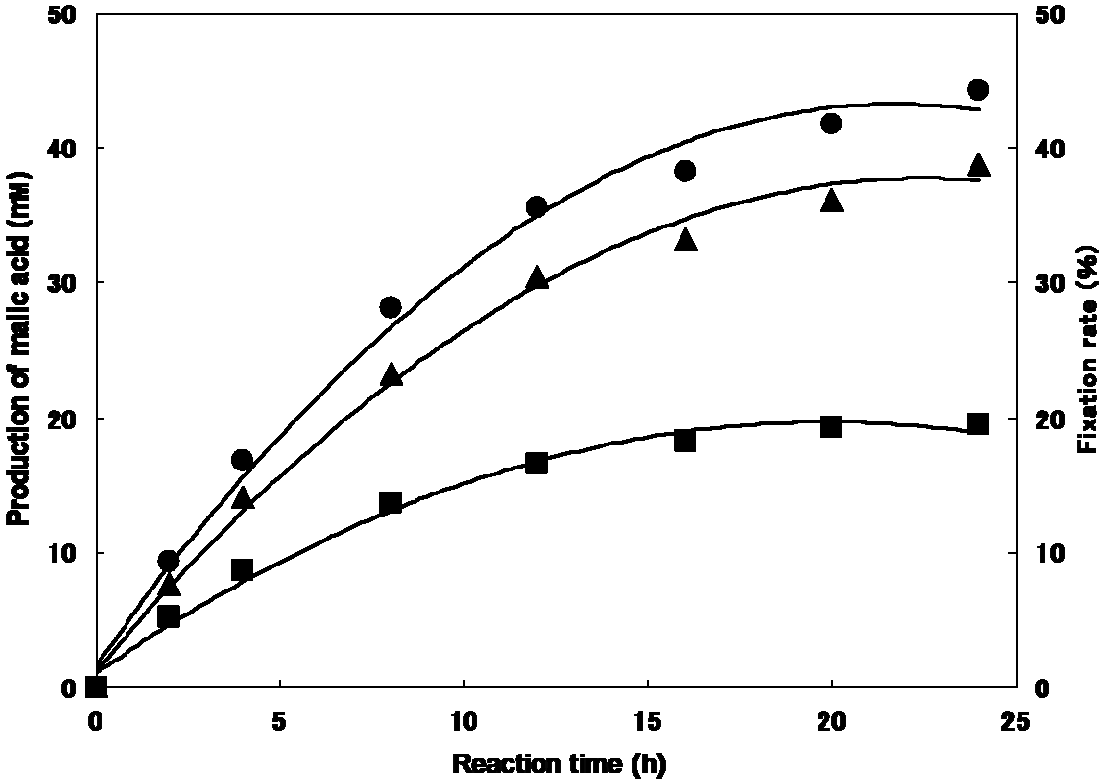

Fig. 10 Effect of NAD^+ concentration on malic enzyme reverse reaction system. Malic acid was measured at 1.0 mM NAD^+ (●), 0.5 mM NAD^+(■), 0.1 mM NAD^+ (▲).

It was already shown that the NAD^+ moieties on Alg- NAD^+ works as an electron transfer carrier in the conjugated two oxidoreductase reaction (36). In the reverse reaction of malic enzyme using conjugated reaction with G6PDH, Alg-NAD^+ also showed improvement of reaction at 100 mM pyruvic acid and 0.1 mM coenzyme concentration in

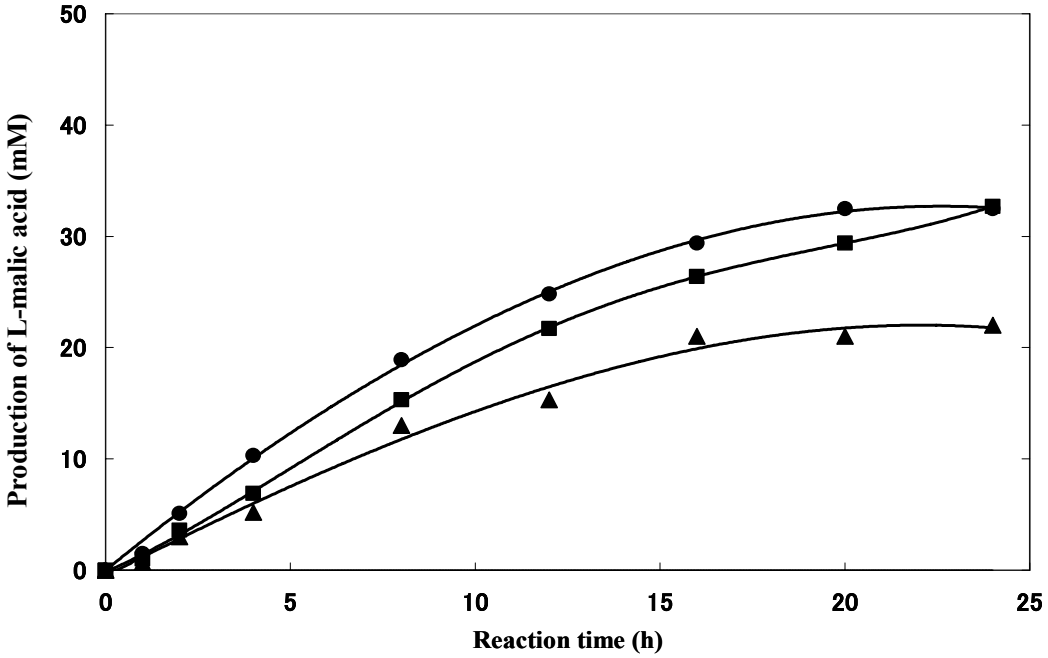

Fig. 11 Malic enzyme reverse reaction system with coenzyme regeneration using Alg-NAD^+.

The reaction was performed under the standard conditions with 1.0 mM Alg-NAD^+ (●), 0.5 mM Alg-NAD^+ (■), 0.1 mM NAD^+ (▲). The other concentrations were the same as for the standard reaction mixture. Concentration of Alg- NAD^+ indicates equimolar concentration of free NAD^+.

the reaction mixture, because NAD cycling number (moles of L-malic acid produce per NAD^+ in the reaction mixture) was calculated to be 200. When 0.1 mM free NAD^+ used for the reaction, NAD cycling number was 76.

CO_2 gas bubbling in water was also fixed into malic acid removing HCO_3- in the system (Fig. 12). The fixation amount of CO_2 was 1.3 mM in 24 h. It was proven that the system works to fix aerial CO_2 into malic acid.

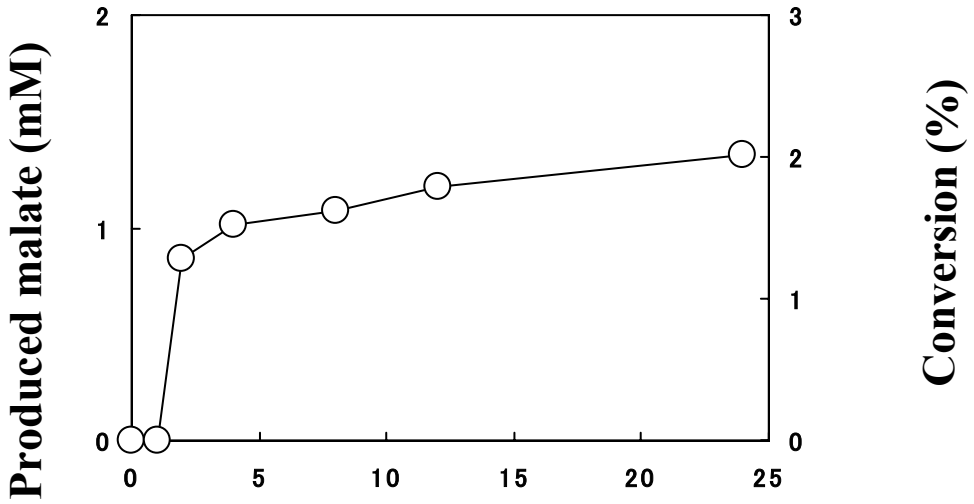

Fig.12 Effect of bubbling CO_2 gas on CO_2 fixation reaction

In conclusion, fixation of HCO_3^- or CO_2 into L- malic acid was successfully carried out in the reverse reaction of malic enzyme of *P. diminura* IFO 13182 with coenzyme regeneration using G6PDH. Recently, several types of enzymatic HCO_3^- fixation using

dehydrogenase reverse reaction have been developed. For example, series reactions of three enzymes (formate dehydrogenase, aldehyde dehydrogenase, and alcohol dehydrogenase) have been applied for HCO_3^- or fixation into methanol (18). Isocitrate dehydrogenase (40) and malic enzyme (11) can also be applied for carboxylation. These results showed 0.007–1.1 mM HCO_3^- fixation. The present work demonstrates 40 mM $KHCO_3$ fixation into L-malic acid, which is about 300 times more efficient than those of the other results. This improvement of the conjugated coenzyme regeneration system warrants further investigation and refinement. Since G6P is expensive and unstable, it is not suitable for practical use. Suye et al. have also developed a modified electrode using a viologen derivative as mediator and Alg-$NAD(P)^+$ immobilized on the electrode surface for efficient NADH regeneration (36). The enzyme reaction conjugated with electrochemical coenzyme regeneration has been successfully carried out. Hydrogen carbonate fixation into pyruvic acid combined with an electrochemical regeneration of coenzyme is studied in the next Chapter.

Summary

Malic enzyme [L-malate: $NAD(P)^+$ oxidoreductase (EC 1.1.1.39)] catalyzes oxidative decarboxylation of L-malic acid to produce pyruvic acid using the oxidized form of NAD(P) ($NAD(P)^+$). We used a reverse reaction of the malic enzyme of *Pseudomonas diminuta* IFO 13182 for hydrogen carbonate fixation into pyruvic acid to produce L-malic acid with coenzyme (NADH) generation. Glucose-6-phosphate dehydrogenase (EC1.1.1.49) of Leuconostoc mesenteroides was suitable for coenzyme regeneration. Optimum conditions for carboxylation of pyruvic acid were examined, including pyruvic acid, NAD^+, and both malic enzyme and glucose-6-phosphate dehydrogenase. Under optimal conditions, the ratio of hydrogen carbonate and pyruvic acid to malic acid was about 38% after 24 h of incubation at 30ºC and the concentration of the accumulated L-malic acid in the reaction mixture was 38 mM. CO_2 gas was also fixed in the same system and the amount was 1.3M, 2% in 24 h. The malic enzyme reverse reaction was also carried out by the conjugated redox enzyme reaction with water soluble polymer bound NAD^+.

Chapter 3 Fixation of HCO_3^- by malic enzyme catalyzed reaction based on regeneration of coenzyme on electrode modified by layer-by-layer self-assembly method

Introduction

Malic enzyme from *Pseudomonas diminuta* IFO 13182 was used for fixation of hydrogen carbonate based on regeneration of NADH by G6PDH catalyzed system in Chapter 2. Although G6PDH system showed NAD/NADH regeneration ability, the cost is too expensive to apply hydrogen carbonate fixation in industrial level. Electrochemical NADH regeneration system was investigated to improve the cost and efficiency for industrial level application in Chapter 3.

This enzyme belongs to NAD-linked dehydrogenase, which catalyzed the reaction as shown in Fig.13. Fixation of hydrogen carbonate was carried out by conjugating with electrochemical regeneration of NAD^+/NADH on carbon plate electrode modified by LBL immobilization of enzyme and mediator (44)

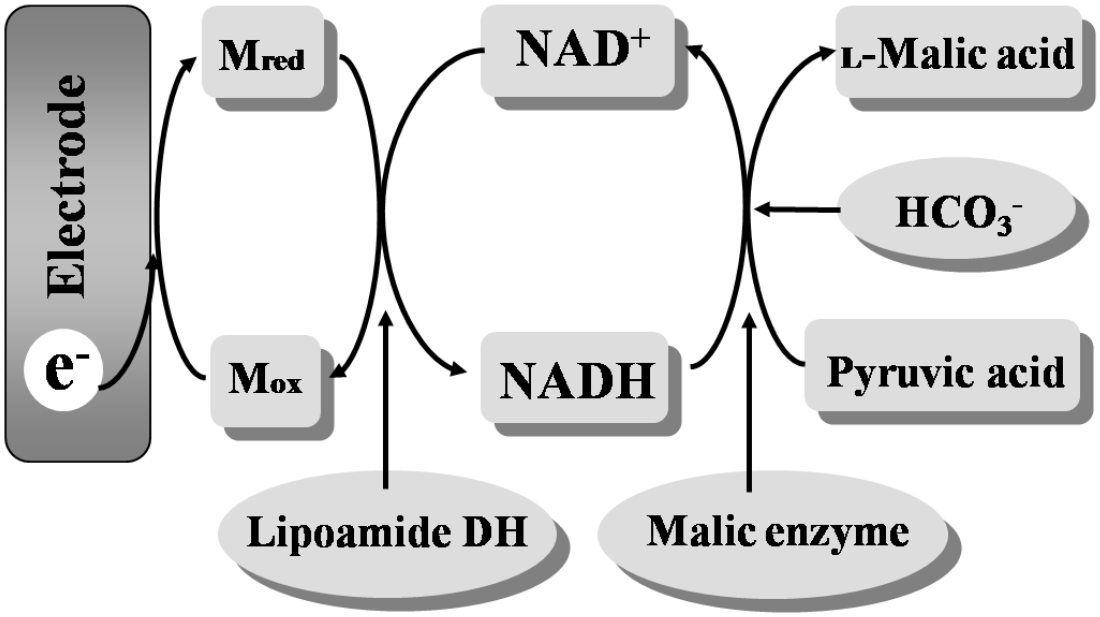

Fig. 13 Fixation of HCO_3^- by malic enzyme catalyzed reaction conjugated with electrochemical NADH regeneration.

Materials and Methods

Chemicals

Sodium alginate (Alg-Na), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), ethylendiaminetetraacetic acid disodium salt (EDTA), L-malic acid and pyruvic acid potassium salt were purchased from Nacalai Tesque Co. (Kyoto, Japan). NADH and NAD^+ were obtained from Kohjin Co. Ltd., (Tokyo, Japan). Polyethyleneimine

(PEI), N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) and lipoamide dehydrogenase (LipDH, EC 1.8.1.4 from porcine heart) were obtained from Sigma Chemical Co. (MO, USA). The other reagents were of analytical grade. Hokuto Denko potensiostat (HA-301, Tokyo, Japan) was used for electrochemical reaction.

Preparation and purification of malic enzyme

Malic enzyme was partially purified from cells of *Pseudomonas diminuta* IFO 13182. Microorganism was grown as described by Suye et al. (23,24). Cells were harvested by centrifugation at 10,000 rpm for 15 min, and then washed with 40 mM phosphate buffer (pH 7.4) containing 10 mM 2-mercaptoethanol and 2 mM EDTA (Buffer A). Washed cells were resuspended in a quarter amount of the Buffer A. The suspension was disrupted with ultrasonic oscillation at 20 KHz for 15 min (UCD-200, Cosmo Biochemical Co., Tokyo, Japan). The cell debris was removed by centrifugation at 10,000 rpm for 15 min. The resulting supernatant was first applied on a DEAE Sepharose CL-6B column (300 x 25 mm, i.d.) previous equilibrated with buffer A. The column was washed with the same buffer and enzyme was eluted with KCl linear gradient of 0-0.5 M in buffer A. The fraction showed

malic enzyme activity was pooled, and the enzyme solution was dialyzed overnight against Buffer A. Then an anion exchange chromatography (HiLoad Q Sepharose column, 110 x 26 mm, i.d.) was carried out. Fraction which showed enzyme activity was collected and then pooled, the enzyme so prepared was referred as partially purified malic enzyme.

Partially purified malic enzyme was used in preparation of immobilized electrode by LBL. Enzyme activity was measured spectrophotometrically, and protein concentration was measured by using Commassie® Protein assay reagent (PIERCE).

Enzyme assay

Activity of malic enzyme forward reaction was determined spectrophotometrically by measuring the increase in the absorbance of NADH at 340 nm. The substrate mixture contained 60 μmol L-malic acid, 30 μmol $MgCl_2$, 0.6 μmol NAD^+, and 100 μmol HEPES-KOH buffer (pH 7.4) in a total volume of 2 ml. The reaction was started by adding 1 ml enzyme solution and the mixture was incubated at 30°C. After exactly 2 min, 1.0 ml of 10% (w/v) sodium dodecyl sulfate (SDS) was added to stop the reaction and the absorbance at 340 nm was measured. In a blank test, 10% SDS was added before the enzyme solution.

One unit of activity was defined as the amount of enzyme that produced one μmol of NADH per min at 30°C.

The reverse reaction of malic enzyme converts pyruvic acid to L-malic acid in the presence of NADH by using $KHCO_3$ as hydrogen carbonate source. The activity was measured spectrophotometrically by measuring the decrease in the absorbance of NADH at 340 nm. The substrate mixture contained 60 μmol of pyruvic acid, 30 μmol of $MgCl_2$, 0.6 μmol of NADH, 60 μmol $KHCO_3$, and 100 μmol of HEPES-KOH buffer (pH 7.4) in a total volume of 2.0 ml. The reaction was started by adding 1.0 ml of the enzyme solution and the mixture was incubated at 30ºC. After exactly 2 min, 1.0 ml of 10% (w/v) SDS was added to stop the reaction and the absorbance at 340 nm was measured. In a blank test, 10% SDS was added before the enzyme solution.

Preparation of polymerized coenzyme (Alg-NAD^+) and polymerized mediator (Alg-V)

The polymerized coenzyme and polymerized mediator were synthesized according to our previous paper (25). Briefly 180 mg Alg-Na and 929 μmol EDC were dissolved in 200

ml distilled water. The final pH was adjusted from 4.5 to 4.7 and the solution was stirred for 60 min at room temperature. After dialyzing with distilled water for 12 h, 929 μmol NAD^+ was added to the solution, and then readjustment of pH to 4.7, resulting solution was stirred for 12 h at room temperature. The mixture was dialyzed sequentially with 10 mM Tris-HCl buffer (pH 7.0) for 12 h, distilled water for 12 h, 2 M NaCl for 24 h and distilled water for 12 h again at 4°C. The product was obtained after freeze drying and referred as Alg- NAD^+. The amount of NAD^+ bounding to the backbone of the polymer was measured at 8.1% and its relative activity was 83%.

Polymerized mediator (viologen compounds) was prepared by the following method. Alg-Na was firstly modified by introducing poly(oxyethylene)diamine by EDC. Then 1-methy-1'-bromobutyl-4,4'-bipyridinum iodide bromide (BrC_4V) was attached on amino groups of the modified Alg-Na, and the polymer so obtained was referred as Alg-V.

Malic enzyme reaction for hydrogen carbonate fixation with electrochemical coenzyme regeneration

Electrochemical regeneration of coenzyme conjugated with malic enzyme reverse reaction for hydrogen carbonate fixation was carried out using two-compartment cell (Fig. 14), which was equipped with three electrode system.

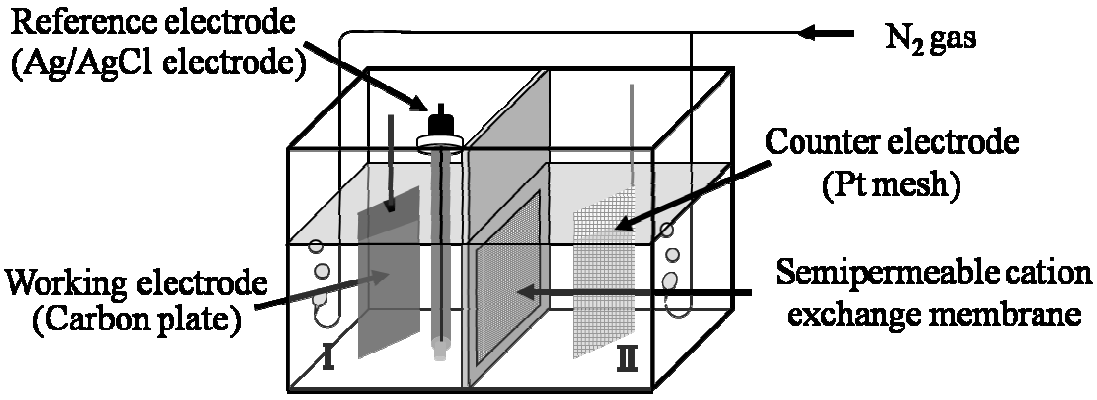

Fig. 14 Electrochemical cell for fixation of hydrogen carbonate.

Both compartments were separated by a semi-permeable membrane (Cellophane membrane) or cation exchange membrane (Neosepta CMB, Tokuyama Soda Co, Tokyo, Japan) (45) and filled with 25 ml of 55 mM HEPES-KOH buffer (pH 7.4). Buffer solution in compartment I also composed of desire amounts of MV^{2+} or Alg-V and 0.1 mM NAD^{+}, 0.1 M $KHCO_3$, 0.5 M pyruvic acid, 2.0 units·ml^{-1} LipDH, and 0.3 units·ml-1of malic enzyme. The cells were immersed in a water bath and continuously purged with high purity nitrogen gas. The objective hydrogen carbonate fixation reaction was conjugated with the

electrochemical regeneration of NADH at a constant potential, -0.9 V vs. Ag/AgCl electrode under 30°C. Produced L-malic acid was measured by high performance liquid chromatography (HPLC) as described below.

Analytical method

Malic acid produced in the reaction mixture was determined by HPLC under the following condition. The separation was carried out on a Shodex®Asahipak ES-502N（100 × 7.6 mm）column with flow rate of 1.0 ml·min-1. UV detector was set at 210 nm, and 0.1 M phosphoric acid solution (pH 4.2) was used as mobile phase. The temperature of column was set at 50°C. Concentration of malic acid was measured by calibration curve method, and fixation rate of hydrogen carbonate was calculated according to the concentration of L-malic acid.

Preparation of modified electrode by LBL method

A carbon plate (25 × 50 × 0.5 mm, Niraco Co., Tokyo, Japan) was used as working electrode, and Pt mesh (25 × 45 mm) and Ag/AgCl served as counter and reference

electrodes, respectively. For LBL immobilization of polymerized coenzyme, polymerized mediator, and enzymes, the carbon plate electrode was firstly cleaned by 0.1 M H_2SO_4, followed by electrochemical oxidation in 10% nitric acid solution containing 2.5% $K_2Cr_2O_7$. A potential of +1.2 V *vs.* Ag/AgCl was applied to bare electrode dipped into the above solution for 10 s, and negative charges (carboxyl groups) were introduced to electrode surface during this operation.

The electrode was then immersed in 1.0 $mg \cdot ml^{-1}$ PEI solution for 20 min, rinsed with Mill-Q water and immersed in 1.0 $mg \cdot ml^{-1}$ Alg-V solution for another 20 min to immobilize the polymerized mediator. A multilayer film could be obtained by repeating the above steps. The same procedure was utilized for multilayer immobilization of LipDH, Alg-NAD^+ and malic enzyme, and the modified electrode was referred as CP/Alg-V n1/LipDH n2/Alg-NAD^+ n3/malic enzyme n4 (n1, n2, n3, n4 denoted the layer number).

Results and discussion

Properties of partially purified malic enzyme from *Pseudomonas. diminuta* IFO 13182

The protein concentration of partially purified malic enzyme was 1.0 mg-protein·ml-1 and exhibited both L-malate decarboxylation activity (forward reaction, 5.0 units·mg^{-1}) and pyruvate carboxylation activity (reverse reaction, 4.5 units·mg^{-1}), respectively.

Fixation of hydrogen carbonate based on electrochemical regeneration of NAD/NADH on bare electrode

NAD^+ can be electrochemically reduced on a bare electrode directly, which needs relatively high over-potential, and inactive dimmer of NAD is produced. On the other hand, enzyme-catalyzed NAD^+ reduction exhibits excellent performance by diaphorase or LipDH in the presence of methyl viologen (MV^{2+}) as mediator (41). In this study, LipDH was used for enzymatic-catalyzed NAD^+ reduction, and both free and polymerized coenzyme, and mediator were used for NADH regeneration and conjugated with the malic enzyme catalyzed reaction for fixation of hydrogen carbonate. Fixation rate of hydrogen carbonate in the presence of free NAD^+ and MV^{2+} was shown Fig. 15. It was found that the fixation

rate increased continuously, and after 80 h the total fixation rate reached at about 40% by using 0.5 mM NAD^+, and 32% in the presence of 0.1 mM NAD^+.

It is inferred that NAD^+ was firstly electrochemically reduced by lipDH catalyzed reaction, and NADH produced was used by malic enzyme catalyzed reaction which

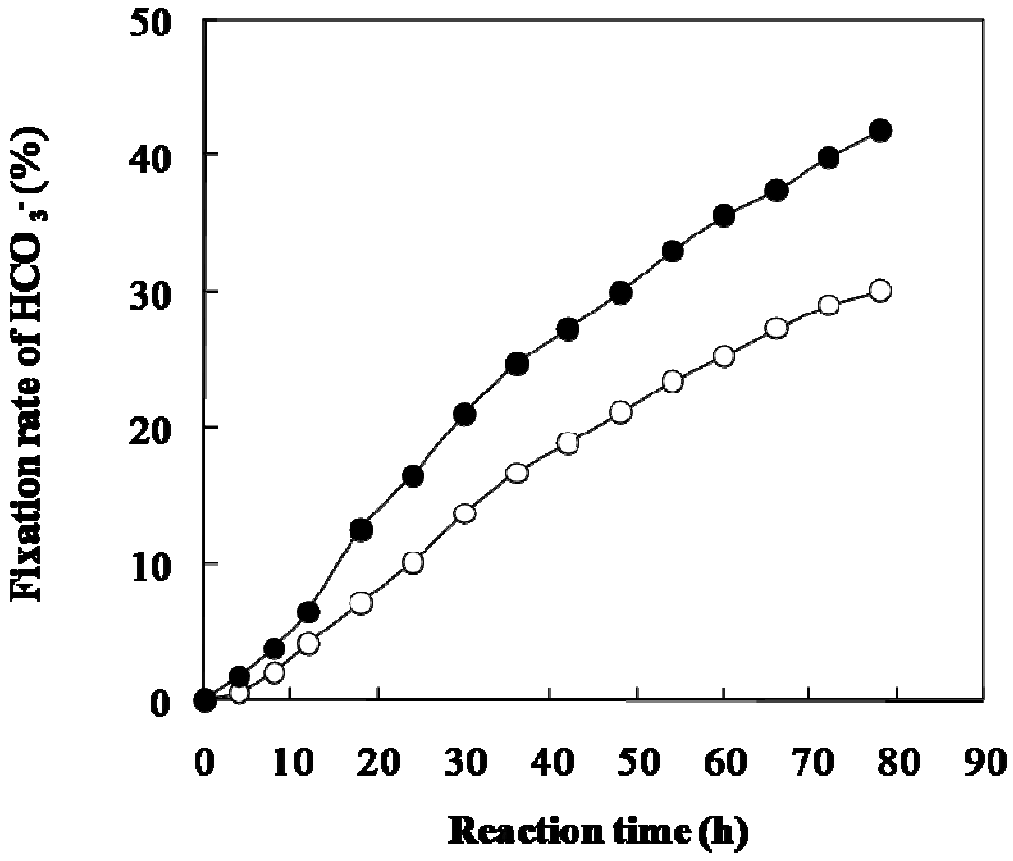

Fig. 15 Time course of hydrogen carbonate fixation by electrochemical reduction in HEPES buffer (pH 7.4) containing 0.1 M $KHCO_3$, 0.5 M pyruvic acid, 2.0 units·ml^{-1} LipDH, and 0.3 units·ml^{-1} malic enzyme in the presence of 0.5 mM NAD^+ and 0.1 mM MV^{2+} (●), 0.1 mM NAD^+ and 0.1 mM MV^{2+} (○) as coenzyme and mediator.

consumed hydrogen carbonate and produced malic acid (Fig. 13). This suggests that NAD^+ can be reduced by LipDH/ MV^{2+} system and coupled with malic enzyme for fixation of hydrogen carbonate. In Chapter 3, both polymerized coenzyme Alg-NAD^+ and polymerized mediator Alg-V were synthesized and their electrochemical characteristics were investigated for regeneration of NADH (25,26), and they were tried here for coenzyme regeneration. A concentration of 0.1 mM Alg-NAD^+ (or 0.1 mM Alg-V) was added in reaction mixture to substitute free NAD^+ (or MV^{2+}), and the performance of polymerized Alg-V and Alg- NAD^+ was shown in Fig. 16.

The fixation rate of hydrogen carbonate reached at about 22% in the presence of 0.1 mM Alg- NAD^+ and 0.1 mM MV^{2+}, and 9.8% in the presence of 0.1 mM Alg- NAD^+ and 0.1 mM Alg-V. Application of polymerized Alg- NAD^+ and Alg-V gave half fixation rate of hydrogen carbonate compared with data by using free NAD^+ and MV2+. Because both coenzyme (NAD^+) and mediator (MV^{2+}) are bound to soluble polyelectrolytes after polymerization, their activities are affected by the high molecular polymer, which resulted in the difference between fixation rates. But Alg-NAD^+ and Alg-V did show an NADH regeneration turnover number of 98, which nearly the same magnitude as that of G6PDH

system (turnover number 75), so there were used in preparation of a modified electrode by LBL method for electrochemical reduction of NAD^+.

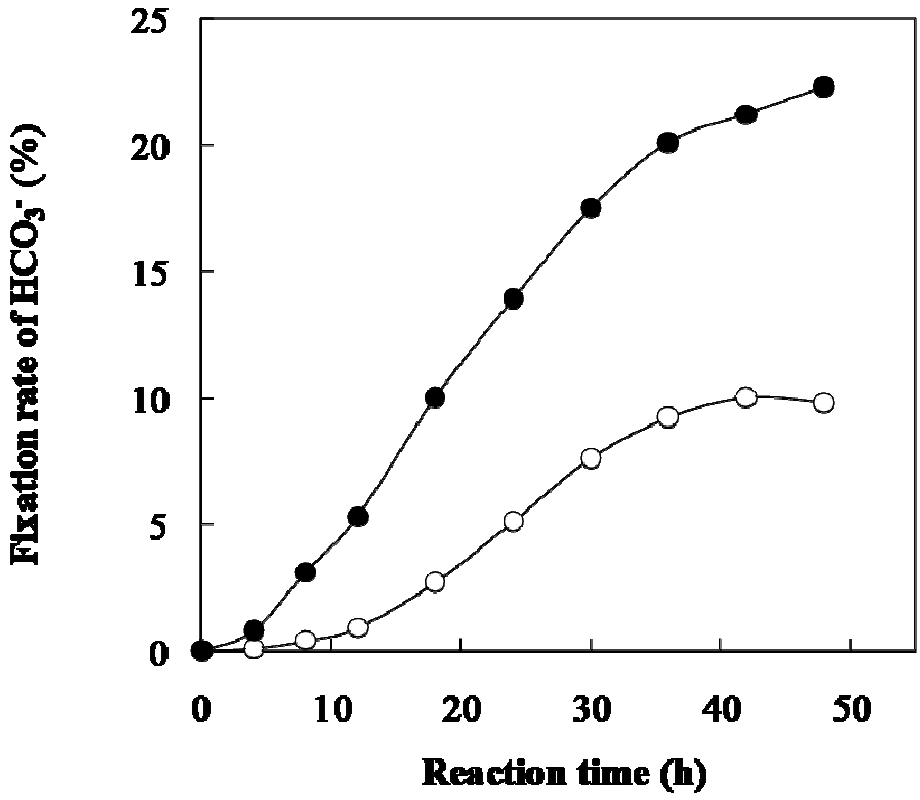

Fig. 16 Time course of hydrogen carbonate fixation by electrochemical reduction in HEPES buffer (pH 7.4) containing 0.1 M $KHCO_3$, 0.5 M pyruvic acid, 2.0 units·ml^{-1} LipDH, and 0.3 units· ml^{-1} malic enzyme in the presence of 0.1 mM Alg-NAD^+ and 0.1 mM MV^{2+} (●), 0.1 mM Alg-NAD^+ and 0.1 mM Alg-V (○) as coenzyme and mediator.

Fixation of hydrogen carbonate on electrode modified by LBL

Because Alg-NAD^+, Alg-V and lipDH in reaction mixture can be applied as electrochemical NAD/NADH regeneration system and be coupled with malic enzyme catalyzed reaction, the modified electrode with immobilization of Alg- NAD^+, Alg-V and LipDH by LBL method was further prepared and used in the above reaction for fixation of hydrogen carbonate.

LBL method is based on the electrostatic force between oppositely charged polyelectrolytes, and has already been develop to the unique technique for immobilization of biomolecules and polymers (42, 43). LBL method, that was used for construction of electrochemical biosensors (27, 28), was applied to carbon fixation in this study. Three electrodes were prepared by immobilizing polymerized mediator, polymerized coenzyme and LipDH respectively, and referred as CP / Alg-V n3, CP / Alg-V n3 / LipDH n3, CP/Alg-V n3 / LipDH n3 / Alg- NAD^+ n3. The fixation rate of hydrogen carbonate on these electrodes by coupling with free malic enzyme were measured and shown in Fig. 13.

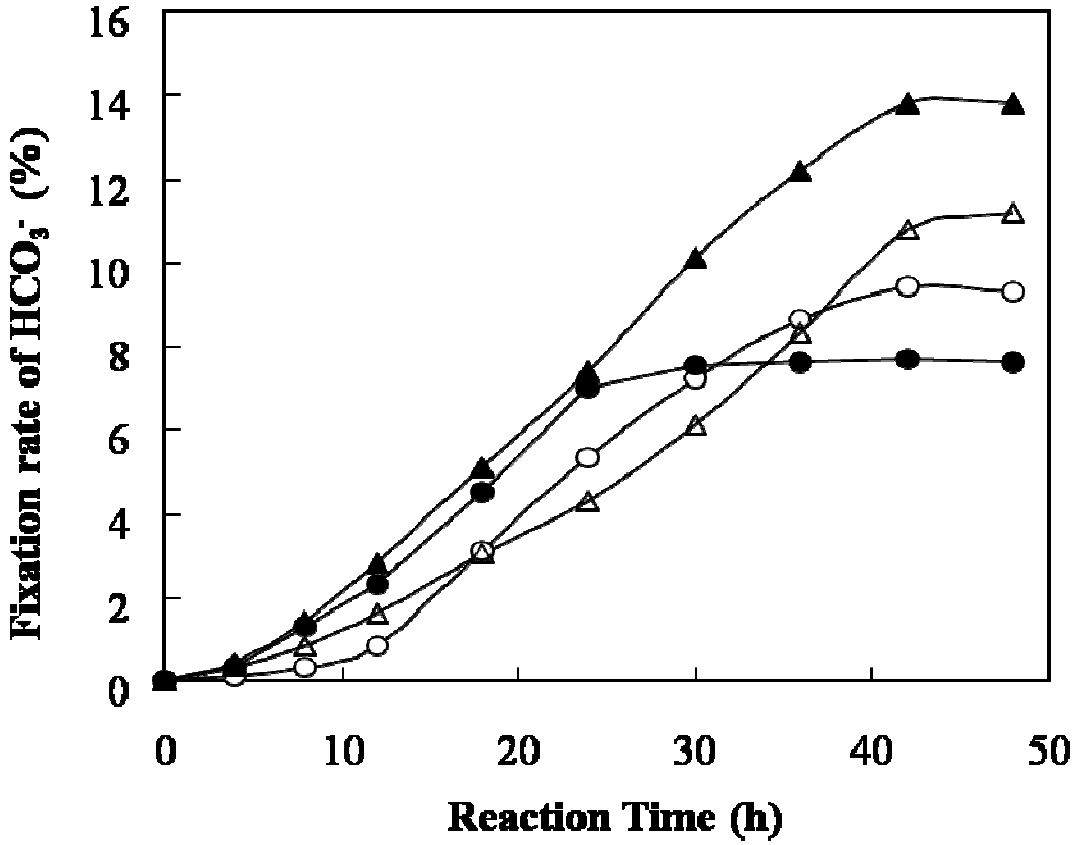

Fig. 17 Time course of hydrogen carbonate fixation in HEPES buffer (pH 7.4) containing 0.1 M $KHCO_3$ and 0.5 M pyruvic acid potassium salt at the following conditions.

A: CP/Alg-V n3, 2.0 units·ml^{-1} LipDH, 0.1 mM NAD^+ and 0.3 units·ml^{-1} malic enzyme (●)

B: CP/Alg-V n3/LipDH n3, 0.1 mM NAD^+ and 0.3 units· ml^{-1} malic enzyme (○)

C: CP/Alg-V n3/LipDH n3/Alg- NAD^+ n3, 0.3 units· ml^{-1} malic enzyme (▲)

D: CP/Alg-V n3/LipDH n3/Alg- NAD^+ n3/malic enzyme n3 (△)

Because polymerized mediator Alg-V in buffer was an efficient mediator as free MV^{2+} in the above experiments, modified electrode with immobilization of three layers of PEI / Alg-V was used for regeneration of NAD / NADH, and reaction mixture contained 0.5 M pyruvic acid potassium salt, 0.1 M $KHCO_3$, 2.0 units·ml^{-1} LipDH, 0.1 mM NAD^+ and 0.3 units·ml^{-1} malic enzyme. After 24 h reaction, fixation rate reached at about 7.6%, which proved that immobilized Alg-V could transfer electron between lipDH and electrode.

LipDH and Alg- NAD^+ was further immobilized by the same LBL method, and gave hydrogen carbonate fixation rate at about 9.3% and 13.8% after 48 h respectively (data shown in Fig. 12), which suggested there was an efficient electron transfer between Alg-V/LipDH/Alg- NAD^+ in multilayer film.

As electrode immobilized with Alg-V, LipDH and Alg- NAD^+ showed NAD/NADH regeneration performance and conjugated with free malic enzyme in buffer for fixation of hydrogen carbonate, three layers of malic enzyme were further immobilized on the electrode by the same LBL method, and electrode so prepared was referred as CP/Alg-V n3 / lipDH n3 / Alg- NAD^+ n3 /malic enzyme n3. Only 0.5 M pyruvic acid potassium salt and

0.1 M $KHCO_3$ were added in buffer for fixation of hydrogen carbonate on such electrode, and a fixation rate of 11.2% was obtained after 48 h as shown in Fig. 17.

It could be inferred that enzymatic reaction occurred according to the reaction scheme in Fig.13, and immobilized coenzyme Alg-NAD^+ in multilayer film on electrode surface could transfer electron between immobilized malic enzyme and substrate. The immobilized amount of NAD^+ bound to Alg-NAD^+ in multilayer film was measured and estimated at about 62 nmol according to Zheng,H. et al (27), and total turnover number of NAD^+ was estimated at about 4500 within 48 h. If monolayer adsorption in each malic enzyme layer was considered, the amount of enzyme on electrode surface is estimated at about 52.5 μg (nearly 0.26 units for 25 ml buffer solution) which is much less than the amount in free enzyme cases (0.3 units·mg^{-1}). It was proven that immobilized malic enzyme retained catalytic activity, and only a very small amount of immobilized enzyme is enough to obtain the same hydrogen carbonate fixation rate as in free enzyme.

Influence of membrane used in electrochemical cell

Two kinds of membranes, semi-permeable membrane (Cellophane membrane) or cation exchange membrane were used in separating the electrochemical cell, and their influence on the fixation rate of hydrogen carbonate was studied. Electrode modified by LBL adsorption of Alg-V, LipDH, Alg-NAD^+ and malic enzyme was applied in buffer containing 0.5 M pyruvic acid potassium salt and 0.1 M $KHCO_3$, and fixation of hydrogen carbonate after 48 h was measured and shown in Fig.18.

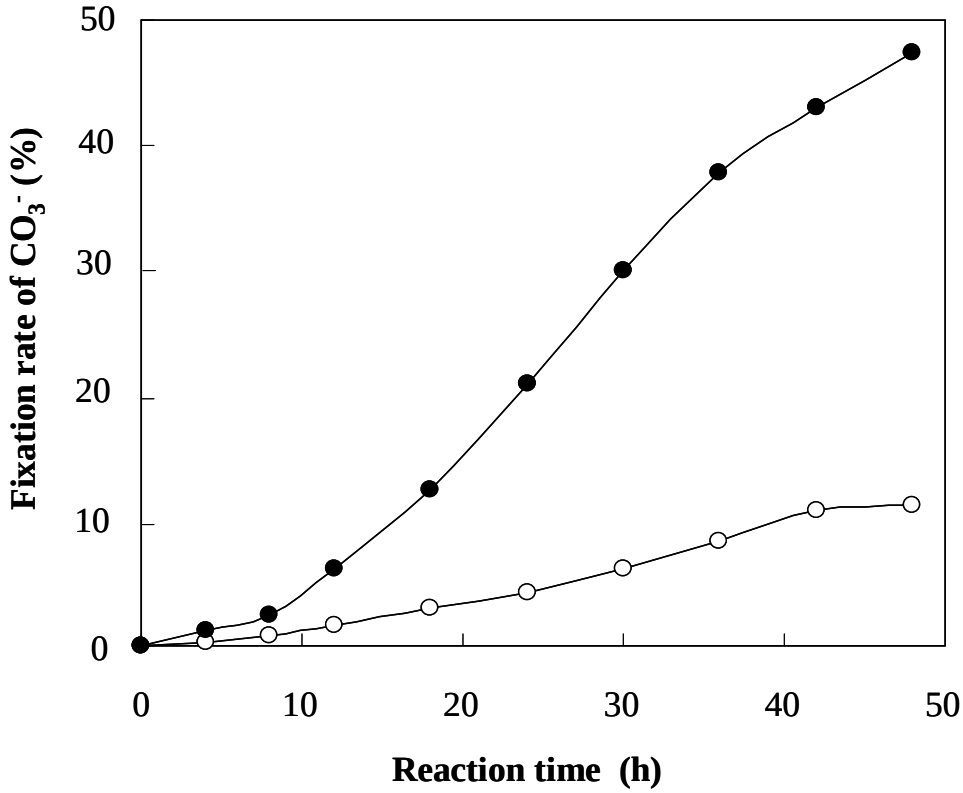

Fig. 18 Production of L-malic acid using the modified electrode.
CP / Alg-V n3 / LipDH n3 / Alg-NAD^+ n3 / malic enzyme n3
with cation exchange membrane (●) and semi permeable membrane (○).

It was found that by using cation exchange membrane, fixation rate reached at 47.4%, which is four times higher than using semi-permeable membrane. The turnover number of NAD^+ obtained in Fig. 14 was calculated about 19000. Cation exchange membrane prevented the reactants to diffuse to the counter electrode, which results in the fast reaction rate in compartment-I and finally the higher fixation rate of HCO_3^- was observed.

Summary

Malic enzyme prepared and purified from *Pseudomonas diminuta* IFO-13182 catalyzed the decarboxylation reaction of malate to pyruvate and HCO_3^- by using NAD^+ as coenzyme, and the reverse reaction was used in this study for fixation of HCO_3^-. The HCO_3^- fixation reaction was based on electrochemical regeneration of NAD/NADH on carbon plate electrode modified by layer-by-layer adsorption of polymerized mediator (Alg-V), polymerized coenzyme (Alg-NAD^+), and lipoamide dehydrogenase (LipDH). Electrochemical reduction of immobilized NAD^+ catalyzed by LipDH in multilayer film

was achieved, and HCO_3^- fixation system with layer-by-layer immobilization of Alg-V / LipDH / Alg-NAD^+ / malic enzyme multilayer film on electrode gave nearly 47.4% of hydrogen carbonate fixation rate in buffer containing $KHCO_3$ and pyruvic acid potassium salt with cation exchange membrane but 11.2% without cation exchange membrane. The total turnover number of NAD^+ within 48 h reached at about 19000, which suggested that an efficient NAD / NADH regeneration and fast electron transfer were constructed within the multilayer film, and the modified electrode can be utilized as a potential approach for fixation of HCO_3^- without addition of free coenzyme.

Chapter 4 General conclusion

The carbon recycle technology in chemical industry was proposed. Malic enzyme [L-malate: $NAD(P)^+$ oxidoreductase (EC 1.1.1.39)] catalyzes oxidative decarboxylation of L-malic acid to produce pyruvic acid using the oxidized form of NAD(P) ($NAD(P)^+$). Reverse reaction of the malic enzyme of *Pseudomonas diminuta* IFO 13182 was used for HCO_3^- fixation into pyruvic acid to produce L-malic acid with coenzyme (NADH) generation.

It was found that glucose-6-phosphate dehydrogenase (EC1.1.1.49) of *Leuconostoc mesenteroides* worked as coenzyme regenerator.

Optimum conditions for carboxylation of pyruvic acid were examined, including pyruvic acid, NAD^+, and both malic enzyme and glucose-6-phosphate dehydrogenase concentrations. Under optimal conditions, the ratio of HCO_3^- fixation in malic acid was about 38% after 24 h of incubation at 30ºC and the concentration of the accumulated L-malic acid in the reaction mixture was 38 mM.

To improve efficiency of HCO_3^- fixation reaction further study was done using electrochemical regeneration of NAD/NADH on carbon plate electrode modified by layer-by-layer adsorption of polymerized mediator (Alg-V), polymerized coenzyme (Alg- NAD^+), and lipoamide dehydrogenase (LipDH). Electrochemical reduction was observed in multilayer film where NAD^+ was immobilized which was catalyzed by LipDH. The system with layer-by-layer immobilization of Alg-V/LipDH/Alg- NAD^+/malic enzyme multilayer film on electrode carried out 47.4% of HCO_3^- fixation rate by using cation exchange membrane in buffer containing $KHCO_3$ and pyruvic acid potassium salt. The total turnover number of NAD^+ within 48 h reached at about 19000, which suggested that an efficient NAD/NADH regeneration occurred and fast electron transfer were constructed inside the multilayer film. The modified electrode is a potential approach for fixation of HCO_3^- saving expensive coenzyme. The LBL method was used to construct electrode for regeneration of NAD/NADH and electrochemical fixation of HCO_3^- . The reaction efficiency was evaluated by fixation rate and NAD^+ turnover number which were summarized in Table 1.

Compared to the other reaction systems, the methods in this study showed much higher HCO_3^- fixation amount, rate and turnover number of NAD^+, especially electrochemical

Table 1. Fixation rate of HCO_3^- （CO_2） with dehydrogenase and coenzyme regeneration

Enzyme	Regeneration	HCO_3^- Fixed anount (mM)	HCO_3^- Fixation rate (%)	Reaction time (h)	NAD^+ turnover number	Reference
Malic enzyme	G6PDH	38 (HCO_3^-) 1.3 (CO_2)	38 2.0	24 24	75 3	Chapter 2
Malic enzyme	Electrochemical (free Alg-V, -NAD^+)	39	39	48	78	Chapter 3
Malic enzyme	Electrochemical LBL method	47	47	48	19000	Chapter 3
Malic enzyme	Photochemical MV^{2+}, FNR	0.65	6.5	3	0.83	Amao et al 2007 (31)
Malic enzyme	Photo chemical	1.1	55	12	11	Inoue et al 1992 (11)
Malic enzyme	Electrochemical MV^{2+}, FNR	0.012	1.2	18	0.12	Sugimura et al. 1990 (9)
Isocitate DH	Electrochemical	0.007	0.07	-	-	Sugimura et al. 1989 (8)
Isocitate DH	photochemical	0.008	0.08	24	-	Inoue et al 1991 (40)
Formate DH	-	0.03	43.8	72	-	Obert and Dave 1999 (18)

fixation of HCO_3^- on carbon electrode with LBL immobilization of Alg-V, LipDH, Alg-NAD^+ and malic enzyme showed remarkable performance. Issue is that the LBL method requires electric power. Photo voltaic cell is being actively developed and clean and low cost electricity is already available. At the next stage it is an interesting subject to test LBL method feeding electron from PV cell.

CO_2 gas was fixed into malic acid with malic enzyme and G6PDH system. IPCC reported that major CO_2 sorces are less than 8,000 facilities in the world(1) (Table 2). High concentration CO_2 gas is available at those facilities. Theoretically malic acid is produced from the free emission gas at the plants using the malic enzyme and G6PDH system. The system also contributes to reduce green house gas.

Malic acid is now industrially made of maleic acid or fumalic acid from oil or natural gas. Malic acid is commonly used as food additive, personal care material, deodorizer, detergent or dye stuff material and recently advertised as supplement to reduce body weight. Polymalate is actively developed as a biodegradable synthetic material for cell culture film, drug delivery system, biomedical materials since poly (β– L-malic acid) is resistant to hydrolysis and can control the degradation speed(46-49). Author is interested in

CO_2 gas fixation rate into malic acid with LBL method feeding the electron from PV cell for future industrial application.

Table 2. Global large stationary CO_2 sources with emissions of more than 0.1 Mt CO_2/year

Process	**No. of sources**	**Emissions ($MtCO_2$/yr)**
Fossil Fuels		
Power (coal, gas, oil and others)	4,942	10,539
Cement production	1,175	932
Refineries	638	798
Iron and steel industry	269	646
Petrochemical industry	470	379
Oil and gas processing	N/A	50
Other sources	90	33
Biomass		
Bioethanol and bioenergy	303	91
Total	**7,887**	**13,466**

(data from IPCC 2005, (1))

Auther is also interested in the other enzymatic CO_2 fixation like acetyl CoA carboxylase or biotinyl enzyme to produce useful chemicals from HCO_3^- or CO_2. The cost effectiveness is a key to develop enzymatic CO_2 fixation for chemical production.

Publication list

Chapter 2

Reverse reaction of malic enzyme for HCO_3^- fixation into pyruvic acid to synthesize L-malic acid with enzymatic coenzyme regeneration.

Yoko Ohno, Toshihiko Nakamori, Haitao Zheng, and Shin-ichiro Suye

Biosci. Biotechnol. Biochem. (2008) **72**, 1278-1282.

Chapter 3

Fixation of CO_2 by malic enzyme catalyzed reaction based on regeneration of coenzyme on electrode modified by layer-by-layer self-assembly method.

Haitao Zheng, Yoko Ohno, Toshihiko Nakamori, Shin-ichiro Suye

J. Biosci.Bioeng. (2009) **107**, 16-20.

Other publication

Co-Immobilization of Malic Enzyme and Alanine Dehydrogenase on Organic-Inorganic Hybrid Gel Fibers and Production of L-Alanine from Malic Acid Using the Fibers with Coenzyme Regeneration.

Koji Nakane, Shin-ichiro Suye, Tomoya Ueno, Yoko Ohno, Tomoko Ishikawa, Takashi Ogihara, and Nobuo Ogata

J. Appli.Poly.Sci., submitted

Oral Presentation

Immobilization of conjugate redox enzyme-system on organic-inorganic hybrid gel fibers and the application to production of amino acid.

Tomoya Ueno, Tomoko Ishikawa, Yoko Ohno, Shin-ichiro Suye, Koji Nakane, Takashi Ogihara, Nobuo Ogata

Abstract of the 16th Joint Conference, The Society of Fiber Science and Technology, Japan , The Textile Machinery Society of Japan and The Japan Research Association for Textile End-Uses (2005)

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